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14. ABSTRACT At the pre-IND conference in 2000 it became clear that the initiation of the clinical trial was to be delayed due to new FDA regulations which required plasmid vectors with kanamycin instead of ampicillin resistance genes, and which discouraged the use of a recombinant adenoviral vector for boosting. This necessitated the development of practically new vaccines and, since the FDA required separate in vitro and in vivo testing, the research has focused on (a) development of the new vaccines; (b) gathering of in vitro data for their safety and efficacy and (c) development of in vivo model for their safety and efficacy. We developed a modified pcDNA3.1 vector that gives high expression in mammalian cells. Dendritic cells transfected with this vector prime to both dominant and subdominant epitopes. Immunization with a construct encoding a xenogeneic protein is superior in breaking tolerance to a self protein. Three immunizations with a cocktail containing the plasmid vaccine and recombinant GM-CSF provide excellent protection against tumor development in a rat model. These results have been incorporated in new IND application.					
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FINAL REPORT

Introduction:

Evidence that the immune system recognizes tumor antigens is supported by the existence of tumor infiltrating lymphocytes but, since cancer cells fail to establish and support an effective immune milieu, tumors often prevail and survive. Worsening the problem is the fact that recognition of cancer antigens on tumor cells seems to evoke a tolerant state by induction of anergy in antigen-reactive T cells. In order to overcome this immunosuppression, tumor-associated antigens can be loaded onto dendritic cells that are not exposed to the microenvironment of the tumor. This can be accomplished in culture with genetic manipulation or pulsing of DC progenitors. Alternatively, patients can be immunized in vivo at a site distant to the tumor with either tumor-associated antigen or with expression vectors encoding for those antigens.

Our approach to immunotherapy of prostate cancer has focused on the use of DNA immunization as a simple, safe and apparently effective form of immunotherapy. Between 1998 and 2000, we conducted a phase I clinical trial using a PSMA plasmid vector and a recombinant adenoviral vector (Ad5PSMA) through collaboration between the Biomedical Research Institute, Rockville, Maryland, and the National Center for Hematology and Transfusion Medicine, the St. Ann Faculty Hospital, and the St. Ivan Rilski Hospital, in Sofia, Bulgaria¹. Sixty-five patients have been accessed into the study and have been repeatedly immunized. Fifty-nine of them have been in the study for a period between 1.5 and 2 years. No patient experienced short or long-term side effects including the development of DNA antibody. In this random population, all patients receiving the optimized protocol became immunized as evidenced by a DTH response and roughly 50% of patients had falling PSA, tumor regression or elimination of bone pains.

Based on these data we proposed and obtained an IRB approval to conduct a clinical trial at the George Washington University Medical Center in Washington, DC.

Body of the report

A. Year 1 of the project (June 1, 2000 – May 31, 2001)

Since we planned to use the same vaccines as described in the original study, an investigational new drug (IND) protocol was submitted to the FDA and a pre-IND teleconference was scheduled for June 2000. At the conference, the FDA recommended that the backbone of the plasmid vaccine be changed to include a bacterial kanamycin-based gene for plasmid selection rather than the ampicillin resistance gene originally used. The myc tag, which we have used until then to detect expression of the encoded protein, was also to be removed. Additionally, since at that time clinical trials involving adenoviral vectors were suspended, the FDA recommended that we did not use such a vector in our clinical trial.

Re-immunization with the viral vector was an essential part of the prime/boost strategy developed in our earlier trials and we needed to investigate other means of re-immunization that will lead to cytotoxic T-cell precursor maturation. The FDA also recommended that, following these modifications, we developed a new in vitro and in vivo animal efficacy models in preparation for a new IND application.

Following the FDA recommendations, in year 1 of the award period we performed the following:

1. Production of rabbit polyclonal sera against the extracellular portion of the human prostate specific membrane antigen (hPSMA).

Designing a new vector that will lead to good expression of the encoded antigen in a mammalian system requires development of methods for its detection after expression in vitro. Since no antibodies that would recognize the extracellular portion of hPSMA were available at that time, and since no sequence for the myc tag was included in the new vector, we needed to produce poly- or monoclonal antibodies against the target antigen. For this purpose we:

- Cloned the sequence encoding for the extracellular portion of hPSMA tagged with poly-His into a bacterial expression vector
- Transfect E-coli with the vector
- Lysed the bacteria following incubation
- Solubilized and purified the recombinant protein using an anti-poly His IgG Sepharose column.
- Used the purified product with incomplete Freund's adjuvant to immunize a rabbit.

For the control experiments, a recombinant PSMA expressed in insect cells was provided by Dr. Jan Konvalinka, Institute of Organic Chemistry and Biochemistry, Czech Academy of Science, Prague, The Czech Republic.

We checked the specificity of the antibodies and whether they reacted with the protein core of PSMA or with the carbohydrate side chains by Western blot. The polyclonal sera reacted with the recombinant PSMA, with the supernatant from COS-1 cells that had been transfected with a variant of vector used in the Bulgarian clinical trial and with a lysate from the human prostate cancer cell line LNCaP (fig.1).

MW Markers

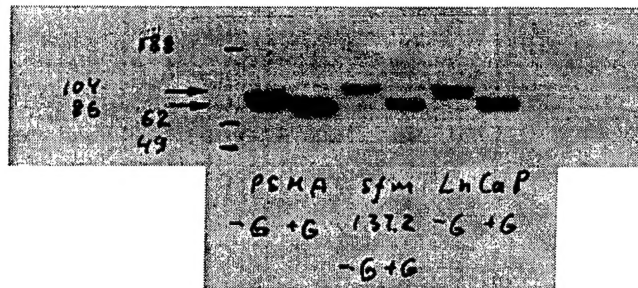


Fig.1. Western blot. Recombinant PSMA (PSMA), serum free supernatant from COS-1 cells transfected with the full length of the human PSMA (sfm) and lysate from the human prostate cancer cell line LNCaP in loading buffer were heated at 70°C for 10 min and loaded on 10% Bis-Tris gels. After electrophoresis and electro-transfer, the nitrocellulose membrane was blocked with 1% casein in TBS/T for 40 min. The membranes were probed with poly- or monoclonal anti-PSMA Abs (see below) for 1 h at RT. Detection was performed using goat anti-rabbit or anti-mouse IgG-HRP conjugates (Sigma) and visualized with WestPico Super Signal Chemo luminescent Substrate (Pierce) in accordance with the manufacturer's recommendations. Legend: -G/+G – in the absence or presence of glycopeptidase F from *Chryseobacterium Meningosepticum* (Sigma) in 50 mM phosphate buffer (pH 7.5) containing 0.1% (w/v) SDS, 50 mM β -mercaptoethanol, 0.75% (v/v) Triton X-100 for 4 h at 37°C.

2. Construction of the kanamycin-based PSMA vector.

The cDNA encoding the extracellular portion (AA 44-750) of the human PSMA (XC-PSMA) was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) after RT-PCR of total mRNA from the human prostate cancer cell line LNCaP (CRL 1740, ATCC). The forward primer introduced a NotI cloning site and a Kozak sequence with a Met codon (GCCACCATG) into the 5'-end of XC-PSMA. The tPSMA plasmid for the transfection experiments was obtained by NotI-XhoI sub-cloning of XC-PSMA into a pVAX1 mammalian expression vector (Invitrogen, Carlsbad, CA). A secretable variant of the tPSMA plasmid – the sPSMA plasmid – was obtained by fusion of the XC-PSMA with a murine Ig k-chain leader sequence. The insert from tPSMA plasmid was sub-cloned by BamHI-XhoI into the mammalian expression vector pSecTag2A (Invitrogen, Carlsbad, CA) providing the murine Igk-chain leader sequence (clone 96). The single SfiI cloning site of the vector was used to fuse the XC-PSMA in-frame with the leader. The 5'-portion of the XC-PSMA between start Met and a single HpaI site was re-amplified in order to introduce SfiI site (Met codon was not included). The SfiI-HpaI fragment of clone 96 was replaced with the PCR product pre-digested with the same restriction endonucleases and the NruI-XhoI fragment from obtained construct was moved to the pVAX1 vector. The inserts in both constructs are under the regulation of a human cytomegalovirus (CMV) immediate-early promoter/enhancer and a bovine growth hormone polyadenylation signal. The plasmid DNA specifications include endotoxin content below 0.1 EU per microgram of DNA; lack of detectable amounts of bacterial RNA, genomic DNA or ssDNA as determined by agarose-gel electrophoresis; less than 10 microgram of

protein per 1 mg of plasmid DNA as determined by colorimetric assay (Bio-Rad, Hercules, CA).

3. Expression of different plasmid vectors (original pcDNA3.1 vs. pVax (kanamycin) vs. pcDNA 3.1(kanamycin substitute) in a mammalian system.

We found a significantly weaker expression of the target antigen when a commercial backbone kanamycin containing vector (pVAX) was compared to a pcDNA3.1 backbone (fig.2).

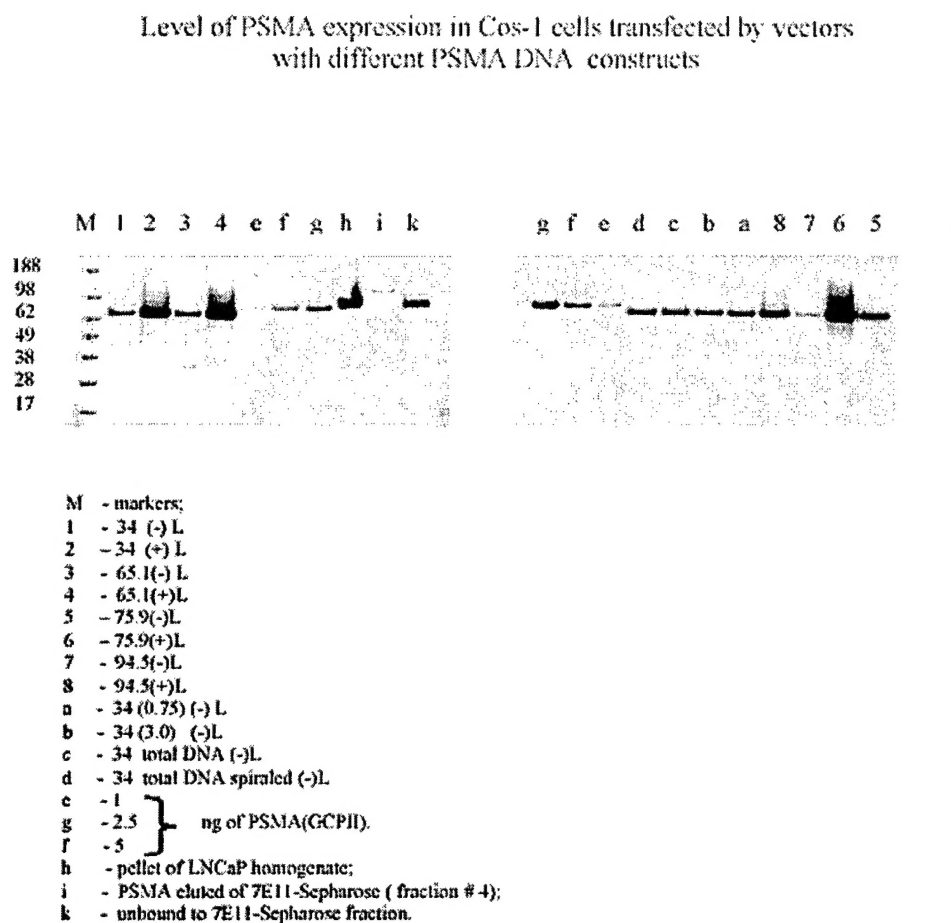


Fig.2. Expression of the extracellular portion of PSMA after transfection with the kanamycin-based backbone (pVAX) plasmid 94.5 (lanes 7 and 8) is weaker when compared to expression after transfection with the original pcDNA3.1 plasmid 34 (lanes 1 and 2) or the PCDNA 3.1 kanamycin substitute 75.9 (lanes 5 and 6). L(+)/L(-) – in the presence or absence of lactacystin (proteasome inhibitor).

For this reason, we decided to work with the pcDNA3.1 kanamycin substitute which we developed.

4. A pre-IND teleconference was scheduled for June 2001.

B. Year 2 of the project (June 2001 – May 2002)

1. Cloning of the rat equivalent of the extracellular portion of hPSMA.

Since re-immunization with the viral vector was an essential part of the prime/boost strategy developed in our earlier trials, we needed to investigate other means of re-immunization that would lead to cytotoxic T-cell precursor maturation. We obtained evidence that immunization with a xenogeneic rather than an autologous protein was much more likely to break existing tolerance. Rat carboxypeptidase II is the rat equivalent of PSMA in the human. We decided to clone this in a plasmid vector and, using a rat model, compare the effectiveness of the “rat” construct (syngeneic) to the “human” construct (xenogeneic) in a model for tumor development prevention. For the R (rat) “PSMA” – K plasmid, DNA encoding for a rat analogue of the human extracellular PSMA was cloned from rat brain tissue and included in a pVax backbone. The insert has 85.66% homology with the human PSMA at the nucleotide level. The R “PSMA”-K plasmid contains a pUC derived pMB1 origin of replication, kanamycin resistance gene and the mammalian transcriptional unit under the regulation of the immediate-early CMV promoter/enhancer and a bovine growth hormone polyadenylation signal. The plasmid size is 5.1 kb.

2. Development of an animal model for safety and efficacy of naked DNA immunization

a) cell proliferation

Standard ^3H thymidine incorporation assays were performed to assess CD4^+ T cell proliferative responses. Splenocytes from immunized rats were resuspended at 5×10^6 cells/ml in RPMI 1640 containing 10% FBS. One hundred microliters was added to each well in 96-well plates with 20, 10, 5, or 0 $\mu\text{g/ml}$ recombinant PSMA (Institute for Organic Chemistry and Biochemistry, Prague, Czech Republic). After 4 days of culture 1 μCi ^3H thymidine (Amersham Biosciences, Piscataway, NJ) was added to each well. Following 16-h incubation, cells were harvested on glass filter paper, and radioactivity was measured in a Beckman LS 2501 liquid scintillation counter. The stimulation index (SI) was calculated as: (cpm with Ag stimulation)/(background cpm without Ag). For experiments involving depletion of CD4^+ or CD8^+ T cells, splenocytes were incubated with magnetic micro beads coated with mAbs specific for murine CD4 (L3T4) or CD8 (Ly-2; Miltenyi Biotec, Auburn, CA). Separation using MiniMACS separation columns was performed according to the manufacturer's instructions. Cell depletions were 95–100% efficient.

b) cell cytotoxicity

Cell Cytotoxicity was determined as previously described ². Briefly, target cells were grown overnight in CM supplemented with 1 with μCi ^3H thymidine (Amersham Biosciences, Piscataway, NJ). The target cells were washed and resuspended in CM. Different target to effector cell ratio were plated in 96 round bottom well plates for 6

hours at 37°C in a humidified incubator. Cells were then harvested on glass filter paper, and radioactivity was measured in a liquid scintillation counter. Cell cytotoxicity was normalized to control samples that contained target cells only and expressed as percent specific lysis.

c) tumor development

Animal weight and tumor development were monitored daily.

d) antibody determination

Western blot. SDS gel electrophoresis was performed using precast Novex gels and buffers. The proteins were transferred onto nitrocellulose membranes (InVitrogen, Carlsbad, CA) in MES (InVitrogen, Carlsbad, CA) transfer buffer, stained with Ponceau red to check the quality of transfer and photographed if necessary. The membrane was then blocked with 1% casein blocker in TBS and stained with primary antibodies (dilutions in TBS, 0.05% Tween20 depending on the antibody) for an hour. The following primary antibodies were used: Y-PSMA1 and Y-PSMA-2 (Yes Biotech Laboratories Limited, Mississauga, Ontario, Canada), rabbit polyclonal anti-GCPII antibodies, anti-GCPII monoclonal antibodies A5, E1, E7, E12 and H2 (developed in the Institute of Organic Chemistry and Biochemistry, Prague, the Czech Republic). Secondary antibody stain, conjugate with horseradish peroxidase (Sigma), dilution 1:10,000 for one hour was performed after three washes with TBS, 0.05% Tween20. Finally, the blots were developed using SuperSignal Chemiluminescent Substrate (Pierce) and exposed to BioMax MR film (Kodak).

In some cases, serum anti-PSMA Ab titers from immunized rats were measured by a direct ELISA. Ninety-six-well plates coated overnight with 100 µl/well of 1 µl/ml recombinant GCPII in PBS were blocked for 2 hours with PBS containing 2% BSA and 0.05% Tween20. Sera were then added in serial dilutions and incubated for 1 hour. The plates were washed three times with PBS containing 0.05% Tween20 and incubated for 1 hour with 1/5000 dilution of a peroxidase conjugated affinity purified anti-rat kappa-lambda chain secondary Ab (Jackson Laboratories, Bar Harbor, ME). The plates were then washed three times, developed with tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, MD), stopped with 1% HCL, and analyzed at 450 nm with a Dynatech MR5000 ELISA plate reader.

d) immunization with a construct encoding human but not rat "PSMA" protects the rats from developing tumors.

Copenhagen rats, inoculated sub-cutaneously with 100,000 syngeneic AT3B1 tumor cells, develop detectable tumors by day 10-12 following injection. To explore the role of pre-immunization on tumor development, rats were immunized three times at 10-day intervals with either saline, empty plasmid vector, H-PSMA or R-PSMA. No rat GM-CSF was included in the immunization cocktail. Fourteen days after the last immunization all animals received 100,000 AT3B1 tumor cells in the right rear flank. Tumors, when developing, were palpable at day 12 following tumor inoculations. Two of the five animals immunized against human PSMA developed tumor. In contrast four of the 5 animals immunized against the rat analogue and in the control

groups developed tumors. The tumors in the group immunized with the human or the rat construct were significantly smaller than the ones in the control group (Table 1). Surprisingly, no protection was seen when the rats were immunized with "secretable" PSMA.

Table 1. Results from a Dunning rat prostate cancer model

Injection with:	Number of animals with tumor (Total number of animals per group)	Mean size of tumor
H"PSMA" K	2(5)	0.5 cm ³
R"PSMA"K	4(5)	1.1 cm ³
S-PSMA	4(5)	1.5 cm ³
Empty vector	4(5)	>2.0 cm ³
Saline	4(5)	>2.0 cm ³

e) addition of rat GM-CSF to the immunization cocktail

In an earlier clinical trial we found that the magnitude of the response could be increased if the plasmid vaccine is co-injected with GM-CSF, a cytokine that has been known for its adjuvant properties. Full protection was observed when recombinant rat GM-CSF was used as an ingredient of the immunization cocktail (Table 2).

Table 2. Results from a Dunning rat prostate cancer model

Injection with:	Number of animals with tumor (Total number of animals per group)	Mean size of tumor
H"PSMA" K +rGM-CSF	0(5)	-
R"PSMA"K +rGM-CSF	0(5)	-
S- PSMA+rGM -CSF	1(5)	1.2 cm ³
Empty vector+rGM- CSF	4(5)	>1.5 cm ³

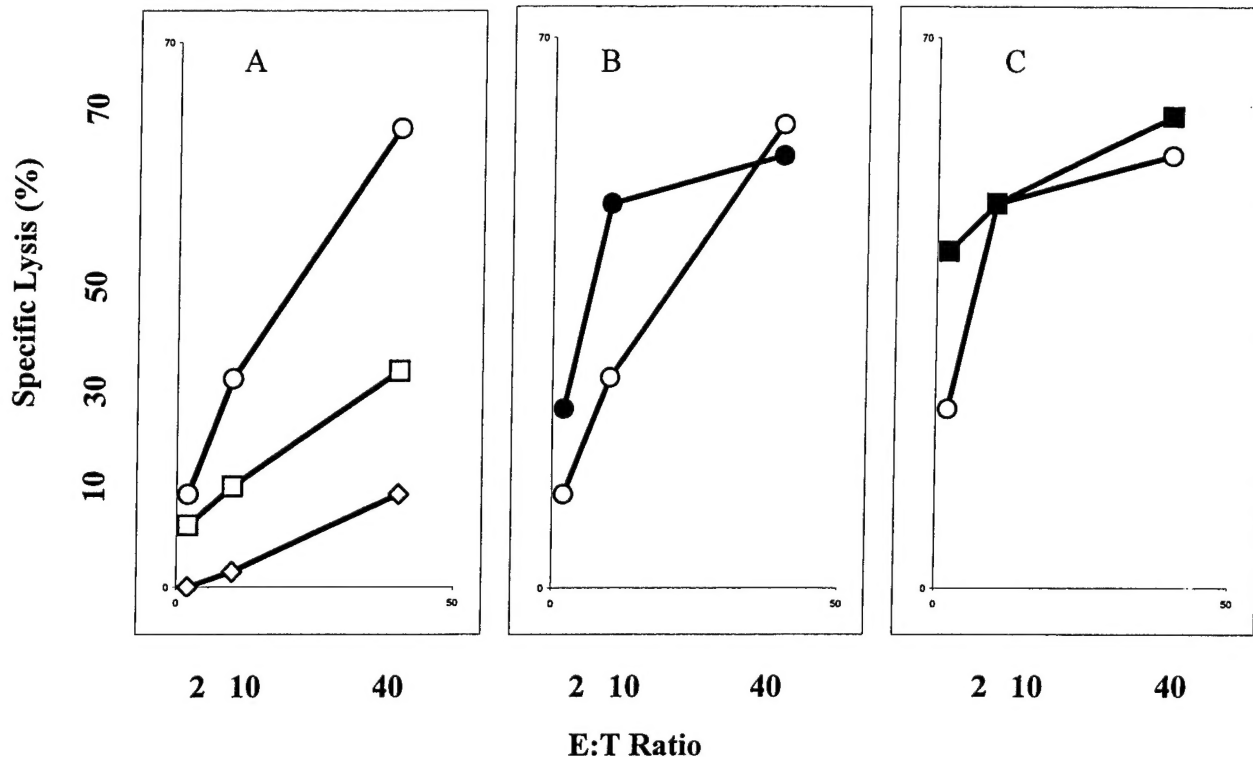


Fig.3. Cytotoxicity of spleen cells from Copenhagen rats that have been immunized against the prostate-specific membrane antigen. A. Rats were immunized with saline (diamonds), empty plasmid backbone (squares), or plasmid DNA encoding for the rat analogue of PSMA (circles). Addition of rat GM-CSF to the immunization cocktail (filled in squares; B-“rat” construct; C-“human construct”) seems to increase the frequency of cytotoxic effectors at lower effector to target ratio.

f) priming with a construct encoding for a xenogeneic protein (“xenogeneic” construct) and boosting with “autologous” construct is most effective in protecting from tumor development.

In an attempt to define the best strategy to induce an immune response against a self-antigen, we explored different priming and boosting strategies. When immunized animals were challenged with 200000 cells, the best protection following immunization involved priming with H-PSMA and boosting with R-PSMA. Surprisingly, good protection was seen following priming and boosting with S-PSMA (fig.4). Spleen cells from these animals gave the strongest proliferative response towards recombinant PSMA (fig.5).

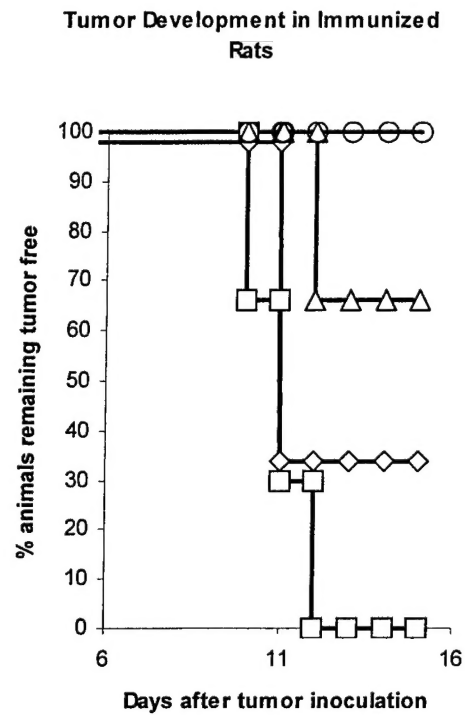


Fig. 4. Tumor development in rats following different prime-boosting regimens. All rats were immunized with a cocktail containing 100 μg plasmid DNA and $9\mu\text{g}/\text{m}^2$ body surface area rat GM-CSF. Circles: Rats were primed with H-PSMA and boosted with R-PSMA; triangles – primed with S-PSMA and boosted with S-PSMA; diamonds – primed with human PSMA, boosted with human PSMA; squares – primed with H-PSMA, boosted with S-PSMA.

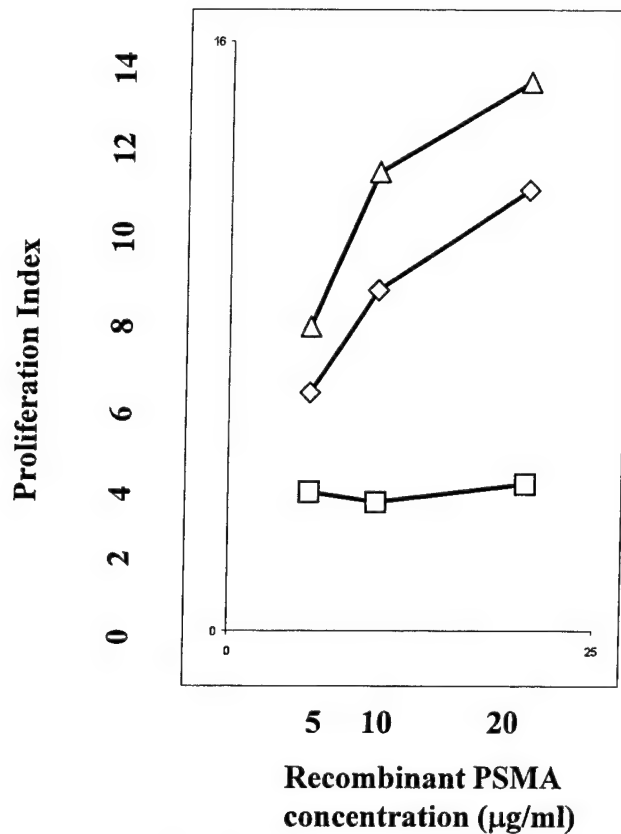


Fig. 5. Proliferation of rat spleen cells after stimulation with recombinant PSMA. Spleens cells from rats immunized with R-PSMA (squares), H-PSMA (diamonds) or S-PSMA (triangles) were stimulated with PSMA. Responses are expressed as proliferation index following normalization to cells that were cultured in complete medium only.

As expected, the immunization with the plasmid encoding the secreted PSMA leads to formation of antibodies against the native protein. The antibodies are of mixed (Th1 and Th2) type (Table 4). Interestingly, priming with the plasmid encoding the truncated form inhibits production of antibodies against native PSMA in the animals boosted with the S-PSMA. At the same time this immunization scheme gives the lowest rate of protection of tumor development (fig.4).

Table 4. Development of antibodies to an autoantigen following immunization with different constructs.

Immunization with	Presence of antibodies against denatured protein core of PSMA	Presence of antibodies against native PSMA	Isotype of antibodies
3x H-PSMA	No	No	N/A
3xR-PSMA	No	No	N/A
3xH-PSMA + rGM-CSF	Yes (very low titer)	No	Not performed
3xR-PSMA + rGM-CSF	Yes (very low titer)	No	Not performed
3xS-PSMA + rGM-CSF	Yes	Yes	IgG1, IgG2a, IgM
H-PSMA; 2xS-PSMA	Yes (very low titer)	Yes (very low titer)	IgG1, IgG2a, IgM

Rats immunized with "truncated" constructs do not develop antibodies towards the native protein. When GM-CSF is used as an ingredient in the immunization cocktail, rats, immunized with the "truncated" constructs develop antibodies towards the denatured protein.

3. At the pre-IND conference, which was held in June 2001, the Center for Biologics Evaluation and Research of the FDA made several suggestions to the design of the experimental animal safety and efficacy trial necessary to support the IND application. They were all taken into consideration and the new design of the animal efficacy model was approved by the Center for Biologics Evaluation and Research. The Center for Biologics Evaluation and Research requested also development of in vitro tests for the potency of the vaccines. An experienced biochemist was hired and potency data for the support of the IND application based on Western blot and ELISA was collected (see year 3 of the project).
4. Since PSMA is expressed on the membrane of endothelial cells that line tumor neovasculature, a vaccine that leads to production of cytotoxic antibodies to this membrane antigen may, in certain cases, be superior or even become a universal anti-cancer weapon. We have now developed a new construct that includes an expression cassette for a "secretable" PSMA (S-PSMA). For the S-PSMA plasmid, the extracellular portion of the human PSMA was cloned into a pSecTag2 vector (Invitrogen), which provided the murine Ig k-chain leader sequence. The insert from the obtained clone was then sub-cloned into a pVAX-1 expression vector (Invitrogen). Three intradermal immunizations of rats with the S-PSMA construct lead to high titer of cytotoxic, complement binding anti-PSMA antibodies that lyse PSMA expressing targets in vitro. We found, however, that animals that were primed with a "truncated" plasmid vaccine never developed antibodies against the native form of the antigen. This is extremely important when secretable tumor markers such as PSA and PAP are used for targets in immunotherapy. Development of antibodies in this case will be of no therapeutic value but will hamper the use of the tumor marker for disease progression. Use of a "truncated" construct for priming will prevent this.

C. Year 3 of the project (June 2002 – May 2003)

The Center for Biologics Evaluation and Research requested also development of in vitro tests for the potency of the vaccines. Our approach in the design of the vaccines has been based on the observation that modified expression cassettes, in which the signal leader sequence or the membrane portion have been eliminated, lead to synthesis of proteins that are not translocated to the endoplasmic reticulum, but are degraded in the proteasome to peptides. Those then become available for MHC presentation and increase the immunogenicity of the vaccine. For this particular reason we have used "truncated" DNA sequences that encode only the extracellular portion of the membrane-bound PSMA. A cytosolic version of PSMA, PSM', however, has recently been described that results from alternative splicing of the PSMA gene. The encoded product has been described to be glycosylated and this, according to the Center for Biologics Evaluation

and Research, contradicted our theory for enhanced proteasomal degradation. To clarify this and to answer the questions asked by the FDA, we designed the following experiments:

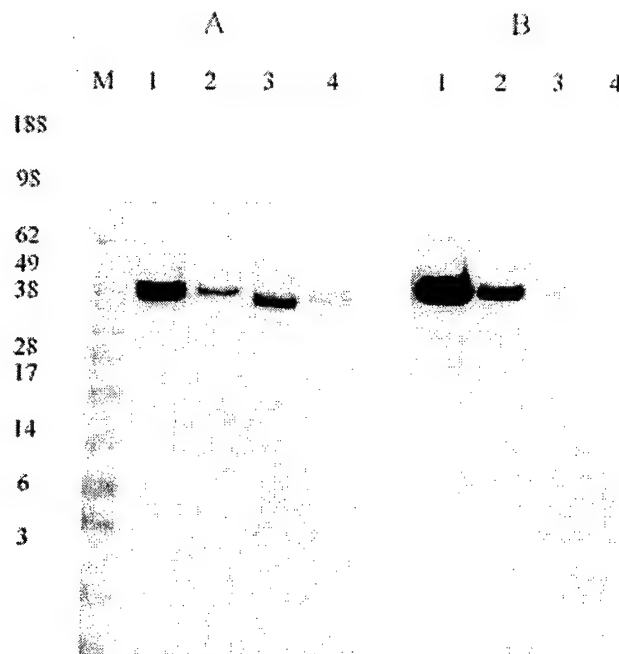
1. Isolation and characterization of PSMA, PSM' and the products expressed following transfection of COS-1 cells with our PSMA plasmid vector:

- **Proteins.** Human prostate specific membrane antigen (PSMA) was purified from LNCaP prostate carcinoma cells as described³. Recombinant human glutamate carboxypeptidase II (GCPII) was provided by Dr. Jan Konvalinka from the Institute of Organic Chemistry and Biochemistry, Prague, The Czech Republic. Positope containing c-myc antigen and 6His tag was obtained from Invitrogen (Carlsbad, CA). Chemical reagents were purchased from Sigma and Fisher Scientific.
- **Antibodies.** Anti-human PSMA monoclonal antibodies Y-PSMA1 and Y-PSMA2 were obtained from Yes Biotech Laboratories Ltd (Mississauga, Ontario, Canada). Anti-myc and anti-His(C-term) monoclonal antibodies were from Invitrogen (Carlsbad, CA). Anti-rat kappa and lambda light chains monoclonal antibodies were purchased from Sigma. Rat immunoglobulin isotyping kit (BD Pharmingen) containing rat immunoglobulin isotype-specific mouse monoclonal antibodies were used for isotyping of anti-PSMA antibodies in rat serum. Rabbit anti-GCPII poly- and monoclonal antibodies were provided by Dr. Jan Konvalinka from the Institute of Organic Chemistry and Biochemistry, Prague, The Czech Republic. Anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated IgGs were purchased from Sigma.
- **Expression and purification of recombinant truncated polyHis tagged PSMA.** Recombinant truncated human polyHis tagged PSMA protein and its rat homologue were expressed in E.Coli BL21 Star (DE3) (Invitrogen, Carlsbad, CA) after transformation with pET28b (Novagen, Madison, WI) vectors coding for extracellular domains of the proteins fused on C-terminus to c-myc/6His tags. Transformation and expression induction were performed according to manufacturer recommendations. Briefly, the culture was grown to optical density A₆₀₀=0.6, induced with 0.3-0.5 mM IPTG and grown for additional 3h at 37°C. After harvesting from 400 ml of LB medium E.Coli cells were resuspended in 20 ml of 20 mM Tris-HCl (pH 8.6) with 150 mM NaCl and complete protease inhibitor (Roche) and disrupted by freezing-thawing. Bacterial DNA was digested with DNaseI (Roche) in presence of 10 mM MgCl₂ during 30 min at 4°C. The lysate was centrifuged (12000 g, 30 min), pellet was washed 3 times with 20 mM phosphate buffer (pH 7.4) containing 2% Triton X-100 and 0.5 M NaCl and dissolved in 5 ml of 8M urea. Recombinant PSMA was recovered from denaturing solution by affinity chromatography on His Trap column (Amersham Pharmacia Biotech). 8M urea protein solution was diluted 1:1 with PBS and applied (30 ml/h) on the Ni⁺⁺ charged HisTrap column equilibrated with PBS. After washing the column with 5 volumes of 5M urea and 5 volumes of PBS with 10 mM of β -octylglycoside (PBS/ β -OG) the protein was eluted with 5 volumes of 0.5M

imidazole in PBS/ β -OG. After elution PSMA was passed through Superose 12 column (Pharmacia Biotech) equilibrated with PBS/ β -OG. PSMA containing fractions detected by Western blot were pooled together and concentrated by ultrafiltration through Centrprep %%% centrifuge filter device. Concentrated PSMA was transferred into 20 mM phosphate buffer (pH 7.4) with 150 mM NaCl by gel filtration through P-10 column and stored at -30°C.

- In vitro expression of PSMA constructs. Expression of PSMA constructs was performed in Cos-1 cells (ATCC). Monolayers were transfected with FuGENE 6 transfection reagent (Roche) and assayed for PSMA production by Western blot. Cos-1 cells were seeded in 6-well tissue culture plates (Nunc, Denmark) at 1.5×10^5 cells per well and grown to 50-70% confluence in DMEM supplemented with 25 mM HEPES (pH 7.5), 1 mM sodium pyruvate, 3.7 g/L sodium bicarbonate, 100 microg/ml penicillin, 100 μ g/ml streptomycin, 0.25 microg/ml amphotericin B and 10% (v/v) of heat inactivated fetal bovine serum. Cos-1 cells were transfected with 1.5 microg of plasmid DNA pre-condensed with 4.5 microl of FuGENE 6 reagent in serum-free DMEM for 30 min at room temperature. Cells were then grown for 72 h in supplemented DMEM and then harvested.
- In proteasome inhibition studies the lactacystin (Sigma) was added to culture media (final concentration 10 microM) 24 h before harvesting. Cells were harvested by gentle scraping, washed twice with 2 ml of cold PBS and lysed in 150 microl of 4% SDS in 125 mM Tris-HCl (pH 6.8) containing 2% of β -mercaptoethanol as described ⁴.
- For detection of secreted PSMA serum containing DMEM was removed 48 h after transfection, the cells in 6-well plates were washed twice with 2 ml of PBS, serum free DMEM (2 ml per well) was added and cells were incubated for additional 24 h. After collection of the medium cells debris were removed by centrifugation (35,000 g, 20 min) and supernatants were concentrated with Centricon centrifuge filtering device (Millipore) and then stored at -30°C.
- The deglycosylation of PSMA proteins was carried out by treatment of 25 μ g samples with 5 units of glycopeptidase F from *Chryseobacterium Meningosepticum* (Sigma) in 50 mM phosphate buffer (pH 7.5) containing 0.1% (w/v) SDS, 50 mM β -mercaptoethanol, 0.75% (v/v) Triton X-100 for 4 h at 37°C.
- Immunoblotting and electrophoretic assays of cell lysates and culture supernatants were performed using NuPage BioTris electrophoretic system (Invitrogen). Protein samples in loading buffer were heated 70°C for 10 min and loaded on 10% Bis-Tris gels. After electrophoresis proteins were electro-transferred onto nitrocellulose membrane and blocked with 1% casein in TBS/T for 40 min. The membranes were probed with poly- or monoclonal anti-GCPII or anti-PSMA Abs for 1 h at RT. Detection was performed using goat anti-rabbit or anti-mouse IgG conjugated with HRP (Sigma) and visualized with WestPico Super Signal Chemoluminescent Substrate (Pierce) in accordance with manufacturer's recommendations.
- Cell surface biotinylation for assay of endocytosis of PSMA by LNCaP cells was performed as described ⁵.

- Isolation of cell membrane and cytosol fractions. Approximately 6×10^7 cells resuspended in PBS (pH 7.4) were disrupted by three fold freezing-thawing and homogenized in glass homogenizer. Plasma membrane and cytosol fractions were prepared by differential centrifugation as described (Howard et al., 1980). Briefly, homogenate was centrifuged at 3000 g (15 min) to remove remaining intact cells, supernatant was re-centrifuged at 10,000 g (30 min) in order to remove the mitochondrial fraction and the collected supernatant was centrifuged at 41,000 g for 30 min for preparation of microsomal (MS) fraction. The precipitate containing MS fraction was resuspended in PBS (pH 7.4) and supernatant was subjected to high-speed centrifugation (140,000 g, 2 h) in order to separate plasma the membrane fraction (PM) present in the precipitate from the cytosol fraction that remains in the supernatant. The PM containing pellet was resuspended in PBS (pH 7.4). All fractions were stored at -30°C
- **Results:**
- Analysis of LNCaP cell homogenate in the presence of protease inhibitors after removing of the microsomal (MS) fraction by differential centrifugation shows that PSMA is localized in plasma membrane (PM) fraction whereas PSM' was found in the cytosol (Fig. 6A). In contrast to previously published data³ only negligible traces of PSMA were detected with monoclonal antibodies 7E11 in the cytosol (Fig. 6, 7). This discrepancy may be explained by more effective removal of membrane fragments from the cytosol fraction in our experiment.

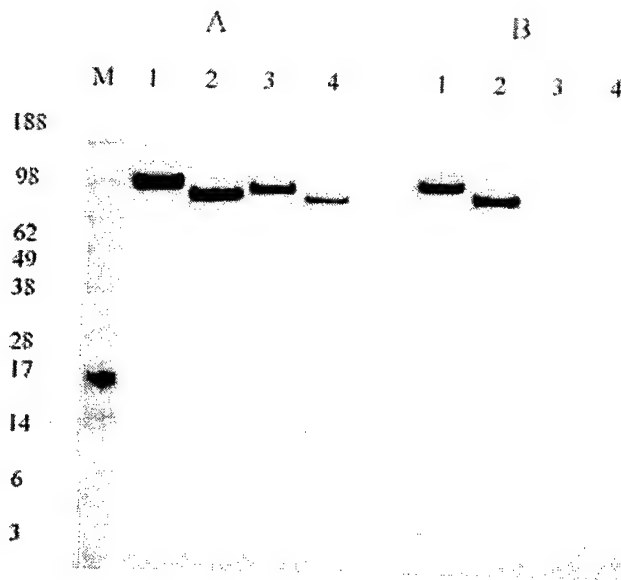


A - probing of nitrocellulose membrane (NC) with anti-GCPII monoclonal antibodies;
 B - probing of NC membrane with 7E11 monoclonal antibody;
 1 - microsomal fraction;
 2 - cell membrane fraction;
 3,4 - cytosol.

Fig.6. Detection of different forms of PSMA in cell membrane and cytosol fractions of LNCaP cells

The MS fraction contained both PSMA and PSM', but PSMA was more abundant (Fig 6, lines A1-2 and B1-2). Thus we found that the MS fraction which is represented mostly by the fragments of ER, contained both PSMA and PSM'. The PM fraction contained PSMA only. The cytosol fraction contained PSM' with traces of PSMA.

- Deglycosylation of PSMA and PSM'. After treatment of the PM and the cytosol fractions with glycopeptidase F, the molecular masses of PSMA and PSM' in the fractions decreased from 103 to 86 kDa and from 96 to 80 kDa respectively (Fig 7). This was interpreted as a sign that both PSMA and PSM' from the PM as well as from the cytosol fractions were glycosylated.



A - probing of nitrocellulose membrane (NC) with anti-GCPII monoclonal antibodies;

B - probing of NC membrane with 7E11 monoclonal antibody;

1 - cell membrane fraction;

2 - cell membrane fraction treated with glycopeptidase F;

3 - cytosol fraction;

4 - cytosol fraction treated with glycopeptidase F.

Fig.7. Deglycosylation of full length and alternative forms of PSMA in membrane and cytosol fractions of LNCaP cells

Glycopeptidase F destroys N-glycosyl bonds and completely deglycosylates both the PSMA and the PSM' proteins. Since the N-type glycosylation activity is shown to be localized in ER, the presence of glycosylated PSM' in cytosol fraction may be explained by two ways:

1. PSM' is translated from alternatively spliced mRNA described earlier ⁶ which codes for a protein that has no apparent signal, intracellular or transmembrane sequences. This protein is translocated into ER by some unknown mechanism and, after glycosylation is translocated back to the cytoplasm.
2. PSM' is a product of degradation of full-length glycosylated PSMA protein during the isolation process.

Assay for PSMA degradation by the cytosol fraction.

PSMA-containing PM-fraction was incubated overnight at room temperature with the PSM'-containing cytosol fraction which was isolated in the absence of protease inhibitors. Detection of PSMA with monoclonal antibodies E711 and Y-PSMA1 showed that incubation of the PM-fraction with or without

added cytosol fraction did not change essentially the intensity or mobility of PSMA bands (Fig 8, lines 5 and 7). Also, the content of PSM' in the cytosol fraction did not change after overnight incubation (detection with Y-PSMA1; Fig 4, lines B4, B6), although the traces of PSMA visible prior to incubation disappeared (detection with E711; Fig. 4, lines A4, A6). The latter may result from non-specific cleavage of the PSMA by cytosol proteases without the formation of stable PSM' molecules. Therefore, we found no evidence that cytosol proteases contributes to specific conversion of PSMA to PSM' during isolation.

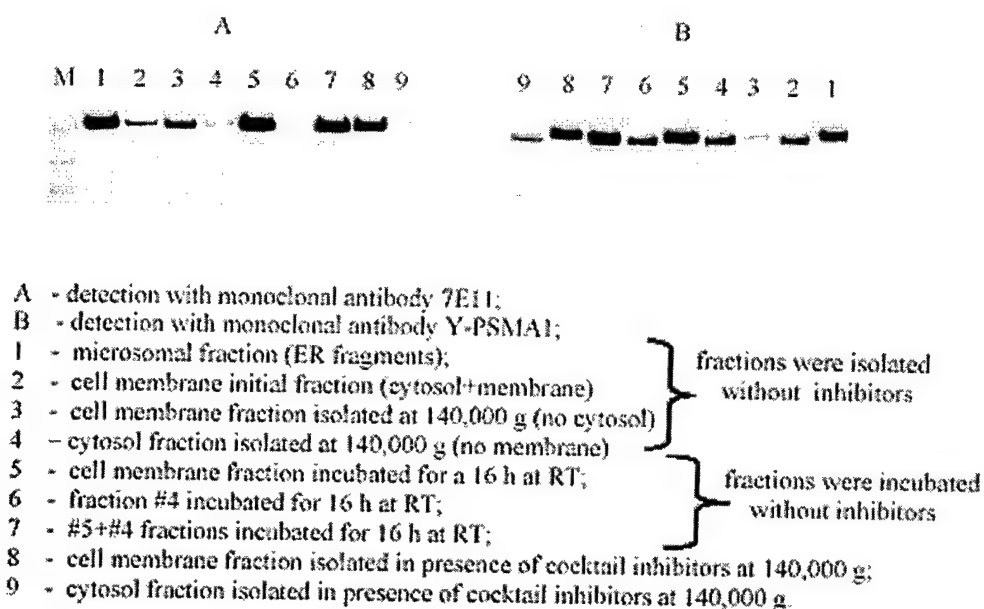
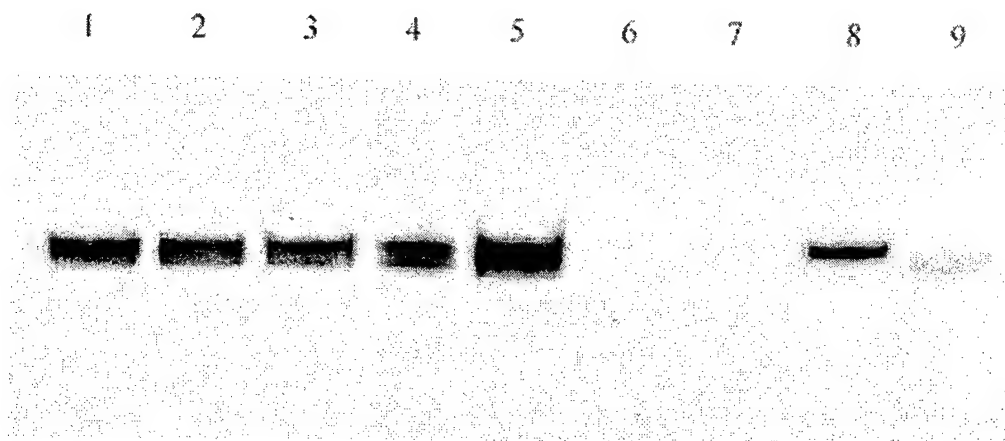


Fig.8. Detection of PSMA in cell membrane and cytosol fractions of LNCaP cells after incubation for 16 hours at room temperature.

- Influence of proteasome inhibitors on PSMA and PSM' expression in LNCaP cells. LNCaP cells were incubated overnight in presence of either lactacystin (10 to 50 μ M), or a mixture of MG101 proteasome inhibitor (Ac-Leu-Leu-norleucinal, 0.1 to 0.5 mM) and cyclosporin A (10 to 50 μ g/ml). The content of PSMA and PSM' in the cell lysates were analyzed visually by Western blot. There is a tendency for an increase of PSM' as the proteasomal inhibition is increased. Finally, at the heights of proteasomal inhibition, the level of PSM' is comparable to the level of PSMA (Fig.9). This offers a second explanation for the existence of PSM'. One may argue that what is observed as PSM' may be a product of PSMA cleavage in the endoplasmic reticulum following its N-type glycosylation. This cleavage yields a glycosylated form that normally constitutes the extracellular portion of PSMA. This newly obtained glycosylated protein is then transported back to the cytoplasm and degraded in the proteasome. It may be detected as PSM' especially if the proteasome is inhibited



- 1 - lysate of LNCaP cells untreated ;
- 2 - lysate of LNCaP cells treated with lactacystin 10 μ M;
- 3 - lysate of LNCaP cells treated with lactacystin 50 μ M;
- 4 - lysate of LNCaP cells treated with Cyclosporin A 10 μ g/ml + MG101 0.1 mM;
- 5 - lysate of LNCaP cells treated with Cyclosporin A 50 μ g/ml+ MG101 0.5 mM;
- 6, 7 - serum containing medium of LNCaP cells treated with Cyclosporin A 50 μ g/ml+ MG101 0.5 mM;
- 8 - plasma membrane fraction of LNCaP cells;
- 9 - cytosol of LNCaP cells.

Fig.9. Expression of PSMA in LNCaP cells treated with lactacystin, MG101 and Cyclosporin A inhibitors. No signal peptide, subcellular or mitochondrial targeting sequences were found in extracellular domain of PSMA using described algorithms⁷⁻⁹.

• Discussion.

After the discovery of an alternatively spliced transcript, PSM', the existence of a shorter variant of PSMA protein was postulated. If coded by PSM', the protein was expected to begin by a Met58. It also should be retained in cytosol since it did not contain any signal for translocation into the ER and, as a consequence, it was expected to be non-N-glycosylated. However, experimental data are in discrepancy with these expectations:

- a) Protein sequence analysis shows that the PSM' lacks the two first amino acids and begins by Ala60.
- b) NAAG-hydrolysing activity in the cytosol from LNCaP and other prostatic tumor cell lines was observed and associated with PSM'^{10,11}, which under conventional wisdom, should be non-N-glycosylated... However, glycosylation of GCPII's extracellular domain seemed to be essential for NAAG-hydrolysing activity¹². Our results also show that, what is detected as a cytosolic form of GCPII (PSM'??) was glycosylated. If the observed NAAG-hydrolysing activity in the cytosol from LNCaP cells is the product of

the already described alternatively spliced transcript, there need to be a new mechanism for translocation to the ER that does not constitute the presence of a signal sequence or a hydrophobic membrane domain. Alternatively, the origin of the intracellular NAAG-hydrolysing activity should come from other products which are not the result of PSM' mRNA translation.

Three possible hypotheses may be formulated to explain these observations:

- a) The glycosylated PSM' is an artifact that results from the cleavage of the full-length PSMA by proteolytic activity present in cytosol during isolation. This hypothesis is not confirmed experimentally since incubation of PSMA (membrane fraction) with the cytosolic fraction in the absence of protease inhibitors does not generate PSM' but only non-specific degradation products.
- b) PSM' is the product of a post-translational cleavage of the glycosylated full-length PSMA in the ER (the full length protein is translocated to the ER, N-glycosylated, the extracellular domain of GCPII is cleaved, the membrane domain is retained in the ER membrane and the soluble PSM' fragment is released in the ER). PSM' is then translocated back to cytoplasm and degraded by the proteasome. If the proteasome is inhibited, the relative quantity of PSM' in cell lysates increases. Experiments with proteasomal inhibition tend to support this hypothesis.
- c) PSM' is the product of modification (proteolytic cleavage?) of glycosylated membrane bound full-length PSMA after its endocytosis from the cell surface. Additional experimentation is necessary to confirm or reject this hypothesis, although the proteolytic enzymes that are present in the lysosomes should be rather degrading the protein but not generate a specific product such as PSM'.

2. In vitro tests for the potency of the vaccines ^{13,14}

- Immunotherapy of prostate cancer could be a safe, non-invasive, relatively inexpensive procedure that can avoid bowel and bladder injury and impotence often resulting from surgical, cryosurgical or radiation therapy. Several groups have recently reported on the safety of DNA vaccines for immunizations against tumor antigens ^{1,15,16}. A possible target for prostate cancer immunotherapy is the prostate specific membrane antigen (PSMA), also known as glutamate carboxypeptidase II (GCPII) ¹⁷⁻²¹. PSMA expression is normally restricted to the prostate gland, brain tissue, jejunum and proximal kidney tubules ²²⁻²⁴. Its expression is increased nearly 10-fold in prostate cancer cells and is also found in tumor but not normal neovasculature ^{20,25}. The main effectors in anti-tumor immunity are CD8+ cytotoxic T cells that recognize tumor-associated antigen-derived peptides in association with major histocompatibility complex (MHC) class I molecules ²⁶⁻²⁸. Gene-based vaccination in its current mode of application is effective in breaking tolerance to a self-antigen, but the response is narrow and is restricted to few of the potential epitopes. This presents a problem in vaccinology since loss of an MHC haplotype that participates in the conformation of the T cell antigen, or point mutation in the recognized sequence would result in ineffective immune surveillance ²⁹⁻³¹. New vaccines and/or new methods of immunizations need to be developed

for those instances. These hopefully will raise responses to subdominant determinants so that the selection of tumor escape variants that fail to express immunodominant epitopes will be prevented²⁹. Numerous factors combine to establish an immunodominance hierarchy³², among them the ineffective generation and transport of sub-dominant epitopes by antigen-presenting cells (APCs). Since proteasomal degradation is the main source of antigenic fragments destined for MHC presentation²⁷, we speculated that purposeful cytosolic retention of newly synthesized tumor-associated antigens in genetically manipulated antigen presenting cells may increase both the quantity and the diversity of such fragments. PSMA is a type II transmembrane glycoprotein which is comprised of 750 amino acids^{33,34}. It lacks a signal peptide sequence and we speculated that elimination of sequences for its transmembrane region might impede the translocation of the encoded product to the endoplasmic reticulum. Such product should not be N-glycosylated, should be retained in the cytosol and rapidly degraded in the proteasome. In theory, DC transfected with such "truncated" sequences, may have an advantage of presenting "subdominant" antigenic determinants that otherwise may not be generated at sufficient density to prime antigen-specific cytotoxic T cell responses. To test these hypotheses, we designed the following experiments for in vitro immunization system with human cells¹³, which compared the ability of two types of vaccines to induce T cell responses to multiple epitopes. The first vaccine (truncated; tPSMA) encodes for only the extracellular domain of PSMA. The product, expressed following transfection with this vector, is retained in the cytosol and degraded by the proteasomes. For the "secreted" (sPSMA) vaccine, a signal peptide sequence is added to the expression cassette and the expressed protein is glycosylated and directed to the secretory pathway. Monocyte-derived dendritic cells (DCs) are transiently transfected with either sPSMA or tPSMA plasmids. The DCs are then used to activate autologous lymphocytes in an in vitro model of DNA vaccination. Lymphocytes are boosted following priming with transfected DCs or with peptide-pulsed monocytes. Their reactivity is tested against tumor cells or peptide-pulsed T2 target cells. Both tPSMA DCs and sPSMA DCs cells generate antigen-specific cytotoxic T cell responses. The immune response is restricted towards one of the four PSMA derived epitopes when priming and boosting is performed with sPSMA. In contrast, tPSMA transfected DCs prime T cells towards several PSMA derived epitopes. Subsequent repeated boosting with transfected DCs, however, restricts the immune response to a single epitope due to immunodominance.

D. Year 4 of the project:

1. In vitro tests for the potency of the vaccines (continued from previous year)

- In a previous study¹³, we found that dendritic cells transfected with products that were cytosolically retained and degraded in the proteasomes, primed autologous T cells to multiple epitopes. Priming with s VacDCs, however, restricted the immune response to one of the epitopes due to immunodominance and the latter was alleviated if anti-CTLA-4 antibodies were present. We wanted to extend these observations to other tumor-associated antigens such as prostate-specific antigen (PSA) and prostate acidic phosphatase (PAP), as well as to study the effect that the removal of CD4⁺CD25⁺ cells prior to priming may have on immunodominance development. Both PSA and PAP are currently used as targets for immunotherapy of cancer³⁵⁻³⁷. On the other hand, gene-based vaccination in its current mode

of application is effective in breaking tolerance to a self-antigen, but the response appears to be narrow and restricted to few of the potential epitopes. For example, the post-vaccination T cell response of some of the HLA A2 patients from the clinical trial performed by us³⁸ was directed against only two of the potential 4 PSMA peptide motifs that had high affinity for binding [M. Mincheff, unpublished]. Immunodominance is a natural mechanism for control that ensures the tight specificity of the immune reaction and prevents untoward autoimmunity, but it also carries the risk of inefficient immune surveillance in cases such as cancer where mutations of the epitope or downregulation of MHC alleles occur^{29,39,40}. Malignant transformation and tumor progression are frequently associated with loss of HLA class I antigens. For example, a recent review of the literature⁴¹ has reported that ~ 15% and 55% of surgically removed primary and metastatic melanoma lesions, respectively, are not stained in immunohistochemical reactions by monoclonal antibodies to monomorphic determinants of HLA class I antigens. Loss or reduced HLA class I antigen expression enables tumor cells to evade the host's immune response^{39,40,42-44} and downregulation of HLA class I antigens in metastases from patients with malignant melanoma has been associated with poorer prognosis⁴⁵. Immunodominance, therefore, presents a problem in vaccinology²⁹⁻³¹. New vaccines and/or new methods of immunizations need to be developed for those instances. These hopefully will raise responses to subdominant determinants so that the selection of tumor escape variants that fail to express immunodominant epitopes will be prevented²⁹. Monocyte-derived dendritic cells are transiently transfected with either sVac or one of two tVacs. The DCs are then used to activate CD25⁺-depleted or non-depleted autologous lymphocytes in an *in vitro* model of DNA vaccination. Lymphocytes are boosted following priming with transfected DCs, peptide pulsed DCs or monocytes. Their reactivity is tested against tumor cells or peptide-pulsed T2 target cells. Both tVacDCs and sVacDCs generate antigen-specific cytotoxic T cell responses. The immune response is restricted towards one of three antigen-derived epitopes when priming and boosting is performed with sVacDCs. In contrast, tVac transfected DCs prime T cells towards all antigen-derived epitopes. Subsequent repeated boosting with transfected DCs, however, restricts the immune response to a single epitope due to immunodominance. While CD25⁺ cell depletion prior to priming with sVacDCs alleviates immunodominance, co-transfection of dendritic cells with GITR-L does so in some but not all cases.

2. Development of an *in vivo* model for testing of safety and efficacy of the vaccines:

- Titration of AT3B-1 and AT3B-1PSA for tumor cell development

Preliminary studies using the Copenhagen rat tumor prostate model showed uniform tumor development in rats that were injected subcutaneously with 100 000 AT3B-1 cells. In humans, the progression of prostate cancer can indirectly be followed by the patient's serum level of the prostate specific antigen (PSA). The AT3B-1 cells do not secrete a rat variant of the human PSA and, in order to have a biochemical marker for the progression of the disease following tumor cell inoculation, we decided to transfect the rat AT3B-1 cell line with the full gene of the human PSA. We needed also to define the tumorigenicity of the PSA-transfected cell line and to compare it to the parental cell line. To do that, healthy, Copenhagen 2331 male retired rat breeders were injected sub-cutaneously in the right flank with different numbers of tumor cells from the AT3B-1 or the AT3B-1^{PSA} rat prostate

carcinoma cell line (see below). For the AT3B-1 cell line, cells (passage 85, delivered 9/03) were purchased from the ATCC. Three rats per group were injected. Injections were performed using 25-ga needles. The animals were observed twice daily for morbidity and mortality, and once daily for clinical signs of toxicity (cage side). A more thorough examination was performed at weekly intervals. Animals were monitored for weight on weekly intervals throughout the whole duration of the study. Tumor size was evaluated every other day by measuring two perpendicular diameters by a caliper. Three days after tumor formation or five weeks after tumor inoculation, all rats were sacrificed, tumor when present were excised and measured. Necropsy was performed, tumor and sera was collected and liver and lungs were inspected macroscopically for metastases.

1. Titration of AT3B-1^{PSA} for tumor induction
 - 5x10⁶ – tumor development in 10-12 days
 - 1x10⁶ – tumor development in 15-19 days
 - 0.500x10⁶ – tumor development 27-29 days
 - 0.250x10⁶ – no tumors
 - 0.1x10⁶ – no tumors
2. Titration of AT3B-1 cells for tumor induction
 - 1x10⁶ – tumor development day 10-12
 - 0.5x10⁶ – tumor development day 10-12
 - 0.25x10⁶ – tumor development day 19-25
 - 0.1x10⁶ – tumor development day 23-25

Conclusion:

1. Injection of the parent cell line (AT-3B1) leads to uniform development of tumors and there is a relationship between number of cell injected and the time of tumor appearance.
2. Injection of 0.25 and 0.1 x10⁶ of PSA-transfected AT3B-1 cells does not result in tumor development.
3. Injection of a larger dose of the transfected cells leads to tumors, but their development is delayed.

For the in vivo model to be proposed to the FDA, all immunized rats will be challenged with 1x10⁶ AT3B-1^{PSA} cells.

- In vivo testing in a rat model the safety and efficacy of a combination of gene-based vaccines encoding human PSMA

The usual dose for DNA immunization of small rodents is 100 ug of plasmid DNA. Safety studies in mice have shown no toxicity in a dose range between 1ug and 100 ug plasmid DNA. After adjusting the dose per body weight, we assumed that injecting of 150 ug plasmid DNA per rat per immunization will have no toxic effect on the animal.

1. Immunization cocktail A: 150 ug H PSMA-T plasmid
2. Immunization cocktail B: 150 ug empty plasmid backbone
3. Immunization cocktail C: saline

Healthy, Copenhagen 2331 male retired rat breeders were used. All rats were immunized three times at 10-day intervals. The immunizations were intradermal and were performed by a standard intradermal injection technique with a small gauge (25-27 gauge) needle and an intradermal bevel. The needle was advanced into the intradermal region and the material was slowly injected while observing the formation of a "bleb" indicative of a proper injection. Volumes of up to 0.1 ml per site were injected.

A total of 104 animals were injected with the immunization cocktails (35 rats with cocktail A, 39 with cocktail B and 30 with cocktail C).

The animals were observed once daily for clinical signs of toxicity (cage side). A more thorough examination was performed at weekly intervals. Animals were monitored for weight on weekly intervals throughout the study. When re-immunizations were performed, mice were re-immunized at weekly intervals. Two weeks after the last immunization, all mice either received a tumor inoculation or were sacrificed, blood and sera were collected and spleens harvested.

All immunizations were tolerated very well and there were no signs of toxicity after immunization. There were no differences in the overall condition of the animals between those who received the gene-based vaccines, the empty plasmid backbone or the saline injections.

Two weeks after the last immunization, all rats were injected sub-cutaneously in the right flank with 1×10^6 tumor cells from the AT3B-1^{PSA} rat prostate carcinoma cell line. Injections were performed using 25-ga needles. The AT3B-1^{PSA} prostate cancer cell line is a non-metastatic cell line. Tumor size was evaluated every other day by measuring two perpendicular diameters by a caliper. The animals were observed twice daily for morbidity and mortality, and once daily for clinical signs of toxicity (cage side). A more thorough examination was performed at weekly intervals. Animals were monitored for weight on weekly intervals throughout the whole duration of the study. Two weeks after tumor inoculation all rats were sacrificed, tumor were excised and measured. Necropsy was performed and kidney, especially proximal tubule, intestines, lungs, liver, prostate and brain were examined by histology. Spleen cells and sera were collected and tested for cytotoxicity against AT3B-1^{PSA} cells or for antibody against the target antigen.

None of the 35 animals immunized with the immunization cocktail A developed tumors. In contrast, 37 of the 39 control rats immunized with the empty backbone and all 30 animals immunized with saline developed tumors 14-20 days after AT3B-1^{PSA} cell inoculation. None of the tumor bearing rats showed distant metastases.

There were no macroscopical or histological changes in the proximal kidney tubule, intestines, lungs, liver, prostate or brain tissues of the immunized rats.

Spleen cells from the immunized animals were cytotoxic against the parent (AT3B-1) and the transfected cell line. The spleens were processed individually. A single cell suspension was obtained cutting and filtering the organ through a sterile cell strainer (70 μ m; Becton

Dickinson, Franklin Lakes, NJ). Thirty million splenocytes were resuspended in 10 ml RPMI 1640 containing 10% heat inactivated FCS, 50 μ M 2-ME, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μ g/ml streptomycin (culture medium) and cultured in a T25 flask with 3×10^6 of mitomycin-c-treated of AT3B-1PSA cells in 10 ml culture medium. After 4 days, blasts were isolated on a density gradient, cultured for an additional day in medium supplemented with 20 IU/ml human rIL-2, and tested for cytolytic activity in a 4-hour 3H thymidine labeled DNA fragmentation assay [3] using the respective targets.

Interestingly, spleen cells from 18 of the 39 animals who were immunized with the empty backbone, and from 19 of the 30 animals who received saline, were cytotoxic against the transfected but not against the parent cell line. We interpret this as evidence that animals become immunized against PSMA after inoculation with the AT3B-1^{PSA} cells. Immunization against PSA could also explain the reduced tumorigenicity of the PSA-transfected cell line (see 2. of the present report) but additional experiments are necessary to clarify this hypothesis.

The presence of anti-PSMA or anti-PSA antibodies in serum pre- and post-vaccination was analyzed by Western blot and ELISA. Anti-human PSA monoclonal antibodies sc-7316 and sc-7638 were obtained from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA). Purified PSA protein was obtained from International Immuno-Diagnostics, Foster City, CA. Anti-human PSMA monoclonal antibodies Y-PSMA1 and Y-PSMA2 were obtained from Yes Biotech Laboratories Ltd (Mississauga, Ontario, Canada). Immunoblotting and electrophoretic assays of cell lysates and culture supernatants were performed using NuPage BioTris electrophoretic system (Invitrogen). Protein samples in loading buffer were heated at 70°C for 10 min and loaded on 10% Bis-Tris gels. After electrophoresis and electro-transfer, the nitrocellulose membrane was blocked with 1% casein in TBS/T for 40 min. The membranes were probed with poly- or monoclonal anti-PSMA Abs (see below) for 1 h at RT. Detection was performed using goat anti-rabbit or anti-mouse IgG-HRP conjugates (Sigma) and visualized with WestPico Super Signal Chemo luminescent Substrate (Pierce) in accordance with the manufacturer's recommendations. Polyvinyl chloride 96-well microtiter plates (Dynatech Laboratories, Chantilly, VA) were incubated overnight at 4°C with a purified preparation of PSA (Vitro Diagnostics, Littleton, CO), as well as BSA or human serum albumin at 100 ng/well in 50 μ l of PBS (pH 7.2). The wells were blocked for 1 h with PBS containing 1% BSA (assay buffer). Tested serum and control pooled rat serum were diluted in assay buffer and added to wells in triplicate in a volume of 50 μ l/well. Purified human antimurine PSA-specific IgG antibody (Fitzgerald Industries, Concord, MA) was used as a positive control for PSA binding. Purified murine IgG was used as a negative control. After incubation overnight at room temperature, the wells were washed four times with assay buffer, and 50 μ l of a 1:4000 dilution of peroxidase-conjugated goat antihuman IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were added to each well. A 1:2000 dilution of peroxidase-conjugated goat antimurine IgG (Kirkegaard & Perry) was used for the PSA antibody control. After incubation at 37°C for 1 h, wells were washed four times with assay buffer, and 100 μ l each of the chromogen O-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) and hydrogen peroxide was added to each well. After a 10-min incubation in the dark, the reaction was stopped with 25 μ l of 4N NH₂SO₄. The absorbance

of each well was measured at 490 nm using an ELISA microplate autoreader (Bio-Tek Instruments, Winooski, VT).

Sera from 17 of the 39 rats animals immunized with cocktail A contained very low titer of antibodies against the target antigens that could be detected on Western against a lysate of COS-1 cells transfected with the respected target antigen. No antibodies against recombinant PSMA or PAP could be detected on ELISA. We interpret this as evidence that no antibodies against the native molecule of the target antigen develop when immunization is performed with the "truncated" HPSMA-T vector whose products are not secreted but proteasomally degraded. In contrast, however, all rats developed antibodies against the native PSA (detectable both on Western blots and ELISA). A possible explanation for this is that the rats become immunized and develop antibodies against the native PSA only after they are inoculated with the transfected tumor cells that secrete human PSA (AT3B-1^{PSA}). Additional support for this hypothesis came from the fact that 22 of the 39 animals immunized with the empty backbone, and 14 of the 30 who received saline, but were all inoculated with AT3B-1^{PSA}, had antibodies against the native PSA. To confirm this observation, the following experiment was performed. Five rats received 3 immunizations with a HPSMA-T vector (pcDNA3.1 vector that encodes for the human PSA without a signal sequence) and were then sacrificed and their sera tested against PSA by Western blot and ELISA. None of the rats developed antibodies against native PSA detected on ELISA. Two of them had low titer of antibodies when tested against a lysate of COS-1 cells on Western blot.

Conclusion:

1. Immunization with cocktail A is safe and well tolerated.
2. Three immunizations with this cocktail provide excellent protection against tumor development induced by inoculation of 1x10⁶ AT3B-1^{PSA} cells.
3. Spleen cells from the immunized rats are cytotoxic to both the parental and transfected cell lines.
4. Spleen cells from some control rats that have been inoculated with AT3B-1^{PSA} cells are cytotoxic to the transfected but not to the parental cell line.
5. Inoculation of rats with AT3B-1^{PSA} cells immunizes them against human PSA. The immune response could be detected as cytotoxicity against the transfected cell line or as antibodies against the native PSA detected by ELISA. This could explain the reduced tumorigenicity of the transfected cell line.
6. Some of the animals immunized with the "truncated" vectors whose products are retained in the cytosol and degraded in the proteasome, contain antibodies to the non-glycosylated forms of the antigen (protein core), but not against the natively folded glycoprotein.

Currently, results from the above experiments are being analyzed and a manuscript is in preparation.

Key Research Accomplishments:

During the award period we have prepared two IND applications and organized two pre-IND conferences with CBER of the FDA. Taken into account all recommendations made by the FDA

Following these recommendations we have:

- a) Re-cloned the extracellular portion of the human PSMA (HPSMA) and its rat equivalent (R-PSMA) in kanamycin-selection based plasmid vectors as recommended by the FDA for a final IND application
- b) Studied the level of expression of the target antigen following transfection of mammalian cells with the said vectors and selection of the highest expressor for in vitro and in vivo studies
- c) Developed an initial animal model for study of the in vivo efficacy of the two plasmid vectors to be used as anti-tumor vaccines in a clinical trial as recommended by the FDA for a final IND application
- d) Developed in vitro evidence for the efficacy of the proposed plasmid vector vaccines to be used in a clinical trial as recommended by the FDA
- e) Studied the cellular localization, possible origin and extent of glycosylation of the PSM' described by others as recommended by the FDA
- f) Developed a final animal model to be tested for in vivo efficacy under GLP conditions for a final IND submission as recommended by the FDA

Reportable Outcomes:

1. IND Application June 2000
2. CDRMP Grant Application for Additional Support for a Prostate Cancer vaccines Clinical Trial, M. Mincheff, PI – May 2000
3. IND Application June 2001
4. Grant Application to NIH Rapid Prevention Intervention Development, M. Mincheff, PI – January 2001
5. Grant Application to the NIH Rapid Action to Intervention Development (RAID) Program, M. Mincheff, PI – March 2001
6. CDRMP Grant Application for Additional Funding for Development of Animal Efficacy Data for prevention of prostate cancer tumor development by DNA vaccines, M. Mincheff, PI – March 2001
7. First Grant Application Resubmission to NIH RAID Program, M. Mincheff, PI – January 2002
8. Second Grant Resubmission to NIH RAID Program, M. Mincheff, PI – June 2002
9. Mincheff M, Zoubak S, Altankova I, Tchakarov S, Makogonenko Y, Botev C, Ignatova I, Dimitrov R, Madarzhieva K, Hammett M, Pomakov Y, Meryman H, Lissitchkov T. Human dendritic cells genetically engineered to express cytosolically retained fragment of prostate-specific membrane antigen prime cytotoxic T-cell responses to multiple epitopes. *Cancer Gene Ther.* 2003 Dec;10(12):907-17
10. Grant application Idea Development Award PC040214, "GENE-BASED VACCINATIONS FOR CANCER IMMUNOTHERAPY", U.S. Army Medical Research

and Materiel Command DOD 2004 Prostate Cancer Research Program; M.Mincheff P.I., submitted 2/04

11. Mincheff M, Zoubak S, Altankova I, Tchakarov S, Pogribnyy P, Makogonenko Y, Botev C, Meryman, HT. Depletion of CD25+ cells from human T-cell enriched fraction eliminates immunodominance during priming and boosting with genetically modified dendritic cells. Cancer Gene Therapy (accepted for publication)

Conclusions:

The initiation of the clinical trial was delayed due to new FDA regulations. The FDA has discouraged the use of a recombinant adenoviral vector for boosting, as well as the inclusion of a myc-sequence to the expression cassette for detection of the successful expression of the target antigen. This necessitated the re-cloning of the target antigen sequence without a myc-sequence into new plasmid backbones and the cloning of the rat equivalent of the human PSMA to be used in the new vaccination scheme. In addition, since the FDA considered the new plasmids new drugs that required separate in vitro and in vivo testing, none of the safety and efficacy data acquired prior to that could be used in an IND application. For that reason, all the research has been directed towards:

- a) development of the new vaccines
- b) gathering of in vitro data for their safety and efficacy
- c) development of an in vivo model for their safety and efficacy to be tested under GLP conditions for an IND application

As a result of these studies the following conclusions have been reached:

- A modified pcDNA3.1 vector that contains a bacterial kanamycin selection gene gives the highest expression in mammalian cells
- Dendritic cells transfected with this vector successfully prime CTL responses in vitro.
- Dendritic cells transfected with a construct whose product is retained in the cytosol and degraded in the proteasome (tPSMA), prime to both dominant and subdominant epitopes. In contrast, sPSMA DCs prime to dominant epitopes only.
- CTLA-4 inhibition during priming in vitro enhances priming to sub-dominant epitopes generated following transfection of DCs with sPSMA.
- In vitro boosting with APCs that express both dominant and sub-dominant epitopes narrows the immune response to the dominant ones.
- Early CD25+ cell depletion during priming in vitro enhances priming to sub-dominant epitopes.
- Co-expression of GITR-L during priming has the potential to alleviate immunodominance.
- Immunization with a construct encoding human but not rat "PSMA" protects the rats from developing tumors.
- Addition of rat GM-CSF to the immunization cocktail improves protection of animals from tumor development

- Priming with a construct encoding for a xenogeneic protein ("xenogeneic" construct) and boosting with "autologous" construct is most effective in protecting from tumor development
- Immunization with the plasmid encoding the secreted PSMA leads to formation of antibodies against the native protein. The antibodies are of mixed (Th1 and Th2) type.
- Priming with the plasmid encoding the truncated form inhibits production of antibodies against native PSMA in the animals boosted with the S-PSMA.
- Rats immunized with "truncated" constructs do not develop antibodies towards the native protein. When GM-CSF is used as an ingredient in the immunization cocktail, rats, immunized with the "truncated" constructs develop antibodies towards the denatured protein.
- The product, obtained from the alternative splicing of the PSMA gene can not be glycosylated. The cytosolic version of PSMA, PSM', is not an artifact (product of degradation of full-length glycosylated PSMA protein during the isolation process). It may be a product of a post-translational cleavage of the glycosylated full-length PSMA in the ER or PSM' is the product of modification (proteolytic cleavage?) of glycosylated membrane bound full-length PSMA after its endocytosis from the cell surface.
- Injection of the AT-3B1 cell line leads to uniform development of tumors and there is a relationship between number of cell injected and the time of tumor appearance.
- Injection of 0.25 and 0.1 x10⁶ of PSA-transfected AT3B-1 cells does not result in tumor development.
- Injection of a larger dose of the transfected cells leads to tumors, but their development is delayed.
- Immunization with the PSMA plasmid/GM-CSF cocktail is safe and well tolerated.
- Three immunizations with this cocktail provide excellent protection against tumor development induced by inoculation of 1x10⁶ AT3B-1^{PSA} cells.
- Spleen cells from the immunized rats are cytotoxic to both the parental and transfected cell lines.
- Spleen cells from some control rats that have been inoculated with AT3B-1PSA cells are cytotoxic to the transfected but not to the parental cell line.
- Inoculation of rats with AT3B-1^{PSA} cells immunizes them against human PSA. The immune response could be detected as cytotoxicity against the transfected cell line or as antibodies against the native PSA detected by ELISA. This could explain the reduced tumorigenicity of the transfected cell line.
- The acquired data successfully answers all the questions posed at the last pre-IND conference with the FDA and can now be incorporated in a new IND proposal.

Appendices:

1. IND Application June 2000
2. CDRMP Grant Application for Additional Support for a Prostate Cancer vaccines Clinical Trial, M. Mincheff, PI – May 2000
3. IND Application June 2001
4. Grant Application to NIH Rapid Prevention Intervention Development, M. Mincheff, PI – January 2001

5. Grant Application to the NIH Rapid Action to Intervention Development (RAID) Program, M. Mincheff, PI – March 2001
6. CDRMP Grant Application for Additional Funding for Development of Animal Efficacy Data for prevention of prostate cancer tumor development by DNA vaccines, M. Mincheff, PI – March
7. First Grant Application Resubmission to NIH RAID Program, M. Mincheff, PI – January 2002
8. Second Grant Resubmission to NIH RAID Program, M. Mincheff, PI – June 2002
9. Mincheff M, Zoubak S, Altankova I, Tchakarov S, Makogonenko Y, Botev C, Ignatova I, Dimitrov R, Madarzhieva K, Hammett M, Pomakov Y, Meryman H, Lissitchkov T. Human dendritic cells genetically engineered to express cytosolically retained fragment of prostate-specific membrane antigen prime cytotoxic T-cell responses to multiple epitopes. *Cancer Gene Ther.* 2003 Dec;10(12):907-17
10. Grant application Idea Development Award PC040214, "GENE-BASED VACCINATIONS FOR CANCER IMMUNOTHERAPY", U.S. Army Medical Research and Materiel Command DOD 2004 Prostate Cancer Research Program; M.Mincheff P.I., submitted 2/04
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